



Expression of genes involved in the embryo-maternal interaction in the early pregnant canine uterus

Kautz, Ewa ; Gram, Aykut ; Aslan, Selim ; Ay, Serhan Serhat ; Selcuk, Murat ; Kanca, Halit ; Koldas, Ece ; Akal, Eser ; Karakas, Kubra ; Findik, Murat ; Boos, Alois ; Kowalewski, Mariusz Pawel

Abstract: Although there is no acute luteolytic mechanism in the absence of pregnancy in the bitch a precise and well-timed embryo-maternal interaction seems to be required for the initiation and maintenance of gestation. Since only limited information is available about these processes in dogs, here, the uterine expression of possible decidualization markers was investigated during the pre-implantation stage (days 10-12) of pregnancy and in corresponding non-pregnant controls. Additionally, the expression of selected genes associated with blastocyst development and/or implantation was investigated in embryos flushed from the uteri used for this study (unhatched and hatched blastocysts). There was an upregulated expression of prolactin receptor (PRLr) and insulin-like growth factor 2 (IGF2) observed pre-implantation. The expression of PRL and of IGF1 was unaffected, and neither was the expression of progesterone- or estrogen receptor (ER). In contrast, ER-levels were elevated during early pregnancy. Prostaglandin (PG)-system revealed upregulated expression of PGE2-synthase and its receptors, EP2 (PTGER2) and EP4 (PTGER4), and of the PG-transporter. Elevated levels of PGF2-synthase (PGFS/AKR1C3) mRNA, but not the protein itself, were noted. Expression of prostaglandin-endoperoxide synthase 2 (PTGS2, COX2) remained unaffected. Most of the transcripts were predominantly localized to the uterine epithelial cells, myometrium and, to a lesser extent, to the uterine stroma. PGES mRNA was abundantly expressed in both groups of embryos and appeared higher in the hatched ones. The expression level of IGF2 mRNA appeared higher than that of IGF1 mRNA in hatched embryos. In unhatched embryos IGF1, IGF2 and COX2 mRNA levels were below the detection limit.

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Title: Expression of genes involved in the embryo-maternal interaction in the early pregnant canine uterus

Short title: Embryo-maternal interaction in the dog.

Authors: E Kautz¹, A Gram¹, S Aslan², SS Ay³, M Selçuk⁴, H Kanca², E Koldaş³, E Akal⁴, K Karakaş², M Fındık³, A Boos¹, MP Kowalewski¹

¹) Institute of Veterinary Anatomy, Vetsuisse Faculty, University of Zurich, Switzerland.

²) Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, University of Ankara, Turkey.

³) Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, University of Ondokuz Mayıs, Samsun, Turkey.

⁴) Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, University of Ondokuz Mayıs, Samsun, Turkey

Correspondence:

PD Mariusz P. Kowalewski, PhD

Institute of Veterinary Anatomy

Vetsuisse Faculty

University of Zurich

Winterthurerstrasse 260

CH-8057 Zurich, Switzerland

Tel.: 0041-44-6358784; Fax.: 0041-44-6358943

Email: kowalewski1@yahoo.de or kowalewski@vetanat.uzh.ch

Abstract

Although there is no acute luteolytic mechanism in the absence of pregnancy in the bitch a precise and well-timed embryo-maternal interaction seems to be required for the initiation and maintenance of gestation. Since only limited information is available about these processes in dogs, here, the uterine expression of possible decidualization markers was investigated during the pre-implantation stage (days 10-12) of pregnancy and in corresponding non-pregnant controls. Additionally, the expression of selected genes associated with blastocyst development and/or implantation was investigated in embryos flushed from the uteri used for this study (unhatched and hatched blastocysts). There was an upregulated expression of prolactin receptor (PRLr) and insulin-like growth factor 2 (IGF2) observed pre-implantation. The expression of *PRL* and of *IGF1* was unaffected, and neither was the expression of progesterone- or estrogen receptor β (ER β). In contrast, ER α -levels were elevated during early pregnancy. Prostaglandin (PG)-system revealed upregulated expression of PGE2-synthase and its receptors, EP2 (PTGER2) and EP4 (PTGER4), and of the PG-transporter. Elevated levels of PGF2 α -synthase (*PGFS/AKR1C3*) mRNA, but not the protein itself, were noted. Expression of prostaglandin-endoperoxide synthase 2 (PTGS2, COX2) remained unaffected. Most of the transcripts were predominantly localized to the uterine epithelial cells, myometrium and, to a lesser extent, to the uterine stroma. *PGES* mRNA was abundantly expressed in both groups of embryos and appeared higher in the hatched ones. The expression level of *IGF2* mRNA appeared higher than that of *IGF1* mRNA in hatched embryos. In unhatched embryos *IGF1*, *IGF2* and *COX2* mRNA levels were below the detection limit.

Introduction

Establishment and maintenance of pregnancy require synthesis and well-orchestrated secretion of a plethora of regulatory factors that establish the uterine milieu needed for embryo implantation and development. The vast majority of these factors remain under the control of progesterone, which is an essential pleiotropic regulator of uterine function.

Because there is no placental steroidogenic activity in the dog, the provision of circulating progesterone depends on corpora lutea (CL) as the major source of this hormone throughout gestation (Concannon *et al.* 1989). In livestock, e.g., cattle, pigs and horses, it is well established that uterine PGF2 α is luteolytic and is responsible for terminating the luteal phase of the estrous cycle in non-pregnant females; therefore, there is no pseudopregnancy in these animals. In contrast to livestock, at least in non-pregnant bitches, there is no uterine luteolysin that could be required for normal ovarian cyclicity, since normal ovarian function is observed following hysterectomy (Olson *et al.* 1984, Hoffmann *et al.* 1992). Furthermore, a luteolytic role of intraluteally produced prostaglandins can be ruled out (Kowalewski *et al.* 2006b, Kowalewski *et al.* 2009). Thus, the absence of an acute luteolytic mechanism in the non-pregnant bitch (Concannon *et al.* 1989, Hoffmann *et al.* 1992) results in a physiological pseudopregnancy and a luteal life span similar to, or even longer than that observed in pregnant bitches. In contrast, in pregnant bitches, the steep prepartum progesterone decline is associated with strongly increased PGF2 α concentrations in the maternal circulation (Nohr *et al.* 1993), implying its role during prepartum luteolysis and/or parturition.

Prostaglandin E2 (PGE2) is one of the important luteotrophic factors in the dog (Kowalewski *et al.* 2008, Kowalewski *et al.* 2009, Kowalewski *et al.* 2013). As recently shown, PGE2 is capable of activating progesterone synthesis in canine luteal cells isolated from early developing CL acting at the level of steroidogenic acute regulatory (STAR) protein expression and function (Kowalewski *et al.* 2013). Although both luteinizing hormone (LH) and prolactin (PRL) are luteotrophic factors, with PRL being the predominant one (Concannon 1980, Okkens *et al.* 1990, Onclin *et al.* 1993, Onclin *et al.* 2000), gonadotrophic support does not seem to be required for luteal maintenance during the early CL phase (Okkens *et al.* 1986).

Consequently, the canine CL seems to possess an inherent life span, resulting in a similar progesterone secretion pattern in pregnant and non-pregnant animals which is mirrored in circulating progesterone levels that do not differ significantly until shortly before parturition; at that time, when a dramatic prepartum progesterone decline is observed, signaling the onset of

parturition (Concannon *et al.* 1989). This hormone profile precludes progesterone as a usable marker for detection of pregnancy in the bitch. Moreover, no pregnancy-associated increase in estrogens is observed in the dog (Hoffmann *et al.* 1994).

Even though knowledge concerning the endocrine control of the canine reproductive cycle has greatly improved, there is still a lack of information concerning the progesterone-dependent establishment of the intimate, initial embryo-maternal contact, and the role of the early canine embryo during this process. In particular, knowledge about endocrine mechanisms regulating the uterine microenvironment prior to implantation is still limited for the dog. This aspect is important for the entire early gestational period up until days 17-18 after mating, at which time implantation takes place in dogs, immediately followed by the start of placenta formation (Amoroso 1952).

Prior to that, the progesterone-dependent decidualization process starts, which is characterized by a very strong, species-specific remodeling of the uterine tissues, especially at the implantation sites. As a result of this change, maternal stroma-derived, so-called decidual cells, are the only cells of the canine placenta expressing the progesterone receptor (PGR) (Vermeirsch *et al.* 2000, Kowalewski *et al.* 2010). Interfering with PGR function, e.g., by application of an antigestagen, will unequivocally lead to preterm parturition/abortion (Baan *et al.* 2008, Kowalewski *et al.* 2010).

In some earlier studies aimed at detecting factors possibly contributing to embryo implantation in the dog, no differences were found in the expression of heat shock proteins and acute phase proteins between the uteri of early pregnant and non-pregnant dogs (Evans & Anderton 1992, Buhi *et al.* 1993, Concannon *et al.* 1996). Recently *CD8*, interleukin (*IL*)4 and interferon gamma (*IFN*γ)-mRNA were found as being abundantly expressed in the early pregnant uterus, while the expression of *CD4*, tumor necrosis factor alpha (*TNF*α) and *IL6* mRNA seemed to be targeted to the non-pregnant uterus (Schafer-Somi *et al.* 2008, Beceriklisoy *et al.* 2009). In contrast to insulin-like growth factor 1 (*IGF1*), the expression of *IGF2* mRNA was found both during early pregnancy and in the non-pregnant uterus (Schafer-Somi *et al.* 2008). Even though these data, which are mostly based on qualitative transcriptional analysis studies, still need further confirmation, they indicate the differential regulation of the uterine function in the pregnant vs. non-pregnant dogs and suggest a possible role of the pre-implantation embryo in this process.

Together with IGF1 and -2, the increased endometrial expression of PRL belongs to the so-called markers of decidualization (Irwin *et al.* 1994, Ramathal *et al.* 2010). Recently, (Kowalewski *et al.* 2011b) we have speculated that PRL acting through endo- and/or paracrine mechanisms might be involved in endometrial glandular secretory function in the dog. Furthermore, Bukowska *et al.* (Bukowska *et al.* 2011) reported an increased expression of *integrins- α 2b*, *- β 2* and *- β 3* and of vasoepithelial growth factors (*VEGF*) *-165*, *-182* and *-188* in the uterus of early pregnant bitches. Nevertheless, the factors and endocrine pathways regulating the functions of the pregnant uterus during the onset of canine pregnancy require further elucidation. Improving our knowledge about the establishment and composition of the proper uterine pre-implantation milieu could improve understanding of the etiopathogenesis of some frequently occurring diestral disorders of the uterus, such as endometrial hyperplasia complex. This disorder seems to originate from a dysregulated endocrinological response of the uterus to hormonal stimulation during the luteal phase of the estrous cycle and is considered by many authors as an initial phase in the development of pyometra. Furthermore, the poor outcome of in vitro fertilization procedures in canids may also be related to an inappropriate oocyte maturation environment, lacking growth factors required for acquisition of full embryo developmental competence (Luvoni *et al.* 2006). Consequently, the expression and cellular localization of several genes that are possibly differentially regulated during canine early pregnancy, including the so-called decidualization markers, were investigated during the pre-implantation stage of pregnancy and in corresponding uterine tissues from non-pregnant dogs. During this early stage of pregnancy, the survival and development of free-floating embryos are dependent on the intra-uterine environment. The expression of E-cadherin (*CDH1*), a cell adhesion protein, whose decreased expression is frequently associated with increased migratory activity of different cell types, was also evaluated. Additionally, while limited by availability of the experimental material, the expression of selected genes was investigated when possible in embryos flushed from the early pregnant uteri used for this study.

Materials and methods

Tissue collections

Uterine tissues from eight (n=8) early pregnant (pre-implantation group, days 10-12 of pregnancy), crossbreed, healthy bitches were used for this study. The day of mating (Day 0) was 2-3 days after ovulation, which was determined by vaginal cytology and by progesterone

measurements (>5ng/ml in peripheral blood). The pre-implantation stage of pregnancy was confirmed by flushing embryos from uteri. Dogs determined as non-pregnant in the uterine flushings served for negative controls (n=6). Uterine samples were collected via ovariectomy. All experimental procedures were performed in accordance with animal welfare legislation.

For isolation of RNA, immediately after surgery, uterine tissues (including all anatomical layers) were trimmed of surrounding connective tissues and shock-frozen in liquid nitrogen; longer storage was at -80°C.

For immunohistochemistry (IHC) and *in situ* hybridization (ISH), after surgery tissue samples were fixed for 24 hrs at +4°C in 10% neutral phosphate buffered formalin. Afterwards, they were washed daily with phosphate buffered saline (PBS) for one week, subsequently dehydrated in a graded ethanol series and embedded in paraffin-equivalent Histo-Comp (Vogel, Giessen, Germany).

Additionally, embryos sampled from 5 uteri (n=19) were available for this study. After careful evaluation under a stereomicroscope, embryos were classified into two groups: hatched blastocysts (n=12, 63%) and unhatched blastocysts (n=7, 37%), and immediately frozen and stored at -80°C.

RNA isolation and reverse transcription (RT)

TRIZOL®-Reagent (Invitrogen, Carlsbad, CA) was used following the manufacturer's protocol in order to isolate total RNA from all samples investigated. The RNA content was measured with a NanoDrop 2000 UV-Vis Spectrophotometer® (Thermo Scientific, Wilmington, USA).

For further purification of the RNA content, DNase treatment with RQ1 RNase-free DNase (Promega, Dübendorf, CH) was performed following the manufacturer's instructions. For each sample, 100-200ng of DNase-treated total RNA were used in the reverse transcription (RT) and complementary DNA (cDNA) was synthesized using RT reagents purchased from Applied Biosystems (Foster City, CA, USA) with random hexamers used as primers according to our previously published protocol (Kowalewski *et al.* 2006b, Kowalewski *et al.* 2011b). All reactions were carried out in an Eppendorf Mastercycler® (Vaudaux-Eppendorf AG, Basel, CH). The following RT conditions were applied: 8 min at 21°C, then 15 min at 42°C, after which the reaction was stopped by incubation for 5 min at 99°C.

Homology cloning of canine specific insulin-like growth factor receptor 1 (IGF1R)

The canine specific *IGF1R* cDNA had not been characterized prior to this study. Thus, to provide required data on the mRNA level, molecular cloning and sequencing were performed. Using an online available predicted sequence, canine-specific *IGF1R* primers were designed and ordered from Microsynth AG (Balgach, CH): forward 5'-CTC GAC AAC CAG AAC TTG C-3' and reverse 5'-GTT GTG GCG GTA AAG GTA AC-3'.

The GeneAmp Gold RNA PCR Kit from Applied Biosystems was used in a hot-start PCR reaction according to our previously described protocol (Kowalewski *et al.* 2006b, Kowalewski *et al.* 2011b). The annealing temperature was 58°C. Total RNA obtained from at least three uterine samples was used, and PCR fragments comprising 717bp of partial canine *IGF1R* were successfully amplified. The following negative controls were run for each experiment: autoclaved water used instead of cDNA (no template control) and the so-called RT-minus control, *i.e.*, samples in which no RT reaction was performed. The PCR products were separated on a 2% ethidium bromide stained agarose gel, extracted using a Qiaex II gel extraction system (Qiagen GmbH Hilden, Germany), subcloned into pGEM-T vector (Promega, Dübendorf, CH) and transformed and amplified in XL1 Blue competent cells (Stratagene, La Jolla, CA, USA). After being purified with Pure Yield™ Plasmid MidiPrep System (Promega), bacterial plasmids were sequenced on both strands with T7 and Sp6 primers (Microsynth). Finally, the cloned sequence was submitted to GenBank with the following accession number: KF793925.

Real Time (TaqMan) PCR and data evaluation

Real time PCR (TaqMan) analysis was performed in an automated fluorometer ABI PRISM 7500 Sequence Detection System (Applied Biosystems), in accordance with the manufacturer's instructions and following our previously described protocol (Kowalewski *et al.* 2010, Kowalewski *et al.* 2011a). The cDNA synthesis and negative controls were as for qualitative PCR described above. Fast Start Universal Probe Master (ROX®) (Roche Diagnostics AG, Schweiz) was used. The semi-quantitation of target gene expression was performed using three independent endogenous reference genes (*GAPDH*, *18SrRNA* and *cyclophilin A*) in the comparative CT method ($\Delta\Delta CT$ method) as described previously (Kowalewski *et al.* 2010, Kowalewski *et al.* 2011a) and according to the ABI 7500 Fast Real Time PCR System manufacturer's protocol. The efficiencies of the PCR assays were established by the CT slope method assuring approximately 100% reaction efficiency. Selected PCR products were sent for

sequencing (Microsynth). Primers and 6-carboxyfluorescein (6-FAM) and 6-carboxytertamethyl-rhodamine (TAMRA)-labelled TaqMan probes, provided by Microsynth, are listed in Table 1. The following canine-specific TaqMan Gene Expression Assays were commercially available and purchased from Applied Biosystems: cyclophilin A (Prod. No. Cf03986523- gH), *E-cadherin* (*CDH1*) (Prod. No. Cf02624268_m1), *IGF1* (Prod. No. Cf02627846_m1) and *IGF2* (Prod. No. Cf02647136_m1).

An unpaired, two-tailed Student's t-test was performed to compare the levels of target genes in uterine samples from early pregnant and non-pregnant control dogs. Numerical data are presented as the mean \pm standard deviation (S.D.). Due to the uneven distribution of the Real Time PCR data obtained for expression of LH-receptor (*LHR*), *IGF1R* and oxytocin receptor (*OTR*), results are presented as geometric means with deviation factor ($X_g \cdot DF^{\pm 1}$). The statistical software program GraphPad 3.06 (GraphPad Software, San Diego, CA, USA) was used. $P < 0.05$ was considered statistically significant.

Immunohistochemistry

Formalin-fixed, paraffin-embedded uterine cross-sections (2-3 μ m thick) from early pregnant and non-pregnant bitches, mounted on SuperFrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany), were subjected to the standard immunoperoxidase detection method following our previously described protocol (Kowalewski *et al.* 2006a, Kowalewski *et al.* 2010, Gram *et al.* 2013a). The list of primary and secondary antibodies and of the respective IgG irrelevant antibodies (negative/isotype controls) is presented in Table 2. Additionally, slides omitting the primary antibodies served as negative controls. The nonspecific binding sites were blocked with either 10% horse serum or 10% normal goat serum, depending on the secondary antibody used in experiments. Peroxidase activity was detected using Liquid DAB+ substrate kit (Dako Schweiz AG, Baar, CH). Sections were counterstained with hematoxylin and embedded in Histokit (Assistant, Osterode, Germany)

***In situ* hybridization (ISH)**

According to the previously described protocol (Kowalewski *et al.* 2006a, Gram *et al.* 2013a), non-radioactive ISH on paraffin-embedded sections was performed in order to investigate the uterine cellular localization of *IGF1* and *IGF2* at the mRNA level.

PCR products generated with the following primers were used for subsequent synthesis of the digoxigenin (DIG)-labeled cRNA probes: *IGF1* (forward) 5'- GGT GGA CGC TCT TCA GTT C -3', *IGF1* (reverse) 5'- TCC TGC ACT CCC TCT ACT TG -3' (product length 268bp, annealing temperature 60°C) and *IGF2* (forward) 5'- GTG CTG CTT TGC TGC TTA C -3', *IGF2* (reverse) 5'- GGG TAT CTG GGG AAG TTG TC -3' (product length 251 bp, annealing temperature 60°C). The DIG-labelled cRNA was detected using alkaline phosphatase conjugated sheep anti-DIG Fab Fragments (Roche Diagnostics) at 1:5,000 dilution in 1% ovine serum according to the manufacturer's instructions. Signals were detected with the substrate 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (NBT/BCIP; Roche Diagnostics).

Results

Temporal expression of selected genes in canine uterine tissues during the pre-implantation stage of pregnancy

Expression of several genes, as listed in Table 1, was investigated at both the mRNA and protein levels in uteri of early pregnant animals (pre-implantation) and compared with their expression in corresponding tissues from non-pregnant dogs (non-pregnant controls).

Expression of all the selected genes was detectable in tissue samples obtained from early-pregnant and non-pregnant dogs. A significantly higher expression of *IGF2* and *PRLr* mRNA was observed in the early pregnant uterus ($P=0.04$ and $P=0.02$, respectively) compared to the controls (Fig. 1). The opposite effect was observed for the uterine expression of *LHR*, which was significantly downregulated during early pregnancy ($P=0.01$) (Fig.1). However, the expression of *IGF1*, *IGF1R* and *PRL* mRNA remained unaffected ($P=0.5$, $P=0.47$ and $P=0.42$, respectively) by uterine exposure to embryos (Fig. 1). In addition, *PRL*-mRNA was generally expressed at a very low level, and was frequently below the detection limit in both groups of animals.

Among the steroid hormone receptors, only the expression of estrogen receptor alpha (*ERα*) was significantly ($P=0.03$) elevated in the early pregnant uterus (Fig. 2), while expression of the progesterone receptor (*PGR*) and of *ERβ* did not differ between the two groups ($P=0.27$ and $P=0.23$, respectively). Similarly, expression of the oxytocin receptor (*OTR*), which varied widely among individuals in both groups, was unaffected ($P=0.5$) by the reproductive status of the animals. The expression of *E-cadherin* (*CDH1*) did not differ ($P=0.25$).

Concerning expression of the major members of the prostaglandin (PG) system, no statistically significant differences were observed for the expression of mRNA encoding for the

prostaglandin-endoperoxide synthase 2 (*PTGS2*, formerly known as *COX2*) (P=0.8), the PGE2 receptor designated as *EP4* (*PTGER4*) (P=0.21), and 15-hydroxy prostaglandin dehydrogenase (*HPGD*) (P=0.29) (Fig. 3 A,D and Fig. 4D). In contrast, a significant upregulation was noted for mRNA expression of PGE2-synthase (*PGES*) (P=0.04) and PGF2 α -synthase (*PGFS/AKR1B3*) (P=0.007), as well as of their respective receptors, *EP2* (*PTGER2*) (P=0.02) and *EP* (*PTGFR*) (P=0.02) and of the PG-transporter (PGT) (P=0.02), in the pre-implantation uterus compared with non-pregnant uterus (Fig. 3B,C and 4 A-C).

Localization of gene expression

In the pre-implantation uterus immunohistochemistry clearly localized IGF2 protein expression to the surface epithelial cells and to the epithelial cells of the superficial and deep uterine glands; clearly detectable signals were also localized in the myometrium. Weaker signals were observed in the endometrial stroma (Fig. 5 B,C). No, or only very weak immunohistochemical signals were observed for IGF2 expression in the uteri of non-pregnant dogs (Fig. 5 A). A localization pattern similar to this but in both early pregnant and non-pregnant uteri was observed at the mRNA level by using ISH (Fig. 5. H,I). As also determined by ISH, *IGF1* expression was co-localized with uterine *IGF2* expression (Fig. 5 H,I). There was no anti-IGF1 canine-specific antibody available for the immunohistochemical studies. The IGF1R protein expression followed the *IGF1* and IGF2 distribution pattern with stronger signals observed in the early pregnant uterus (Fig. 5 D,E). While weaker endometrial signals were observed for PRLr protein expression in non-pregnant animals (Fig. 6 A,B), stronger signals were detected in the surface and glandular endometrial epithelial cells of the early pregnant uterus (Fig. 6 C,D). Clear myometrial staining was noted in both groups of animals (Fig. 6 B,D).

Expression of PGR was detectable in the uteri of both early-pregnant and control animals but did not vary widely between the two groups. The immunohistochemical signals were localized to nuclei of both superficial and glandular epithelial cells, as well as the smooth muscle cells of the myometrium and to a lesser extent in the endometrial stroma (Fig. 7 A,B).

The ER α protein was co-localized with PGR, however, signals were distinctly stronger in uteri after embryo exposure than in the non-pregnant controls (Fig. 7 C,D). Much weaker staining was observed for uterine ER β expression which did not differ between the two groups and showed a similar localization pattern as for the other nuclear receptors (Fig. 7 E,F).

Whereas endometrium stained negatively for COX2, however, myometrial signals were strong (Fig. 8 A), but no or only very weak uterine signals were observed for PGFS/AKR1C3 protein, in both groups of animals (Fig. 8 B).

The expression of PGT, PGES, EP2 and EP4 revealed a similar protein distribution pattern as PRLr, showing their co-localization and higher abundance in endometrial epithelial cells, the myometrium and stromal cells in the pre-implantation uterus (Fig. 8 C,D and 9 A-F). For all these factors, staining in the endometrial stroma was weaker than in the epithelial compartments. High variability for the HPGD immunohistochemical signals was observed between individual animals in both groups. They tended, however, to be stronger in the early-pregnant uteri and revealed a similar localization pattern, but with distinctly stronger staining in the deep uterine glands (Fig. 8 E-G).

Expression of selected genes in early, free-floating canine embryos prior to implantation

Due to the limited availability of the embryo material, investigations were restricted to detecting the expression of *IGF1*, *IGF2*, *COX2* and *PGES* in the two groups of embryos (unhatched and hatched blastocysts) collected from five early pregnant bitches. Embryos were pooled in order to reach the required limits of detection. Consequently, no statistical analysis of gene expression was possible.

Whereas the expression of *IGF1*, *IGF2* and *COX2* mRNA was below the detection limit in the unhatched embryos, the *PGES* mRNA was abundantly expressed and detectable in both unhatched and hatched blastocysts, with apparently higher expression level in the latter ones (Fig. 10A). The expression of *IGF2* mRNA seemed to be higher than that of *IGF1* mRNA in the hatched group of embryos (Fig. 10 B), while *COX2* and *PGES* showed similar transcript abundance (Fig. 10 C).

Discussion

The uterine response to early embryo exposure was investigated during the pre-implantation stage of canine pregnancy by measuring the expression of several target genes. Our investigations were based on the assumption that, in view of the lack of an anti-luteolytic signal in the dog resulting in similar hormonal status in early-pregnant and non-pregnant dioestric bitches, some local effects would be exerted by the early pre-implantation embryo that modify the uterine milieu, serving as a prerequisite for a successful implantation and ensuring embryo survival prior

to attachment. The effects of seminal plasma-derived bioactive factors in modulation of the uterine endocrine milieu, and possibly having an impact on the initiation of pregnancy in dogs, were not separately investigated in this study, but would certainly merit future investigations.

Among the genes investigated, IGF1, IGF2 and PRL count as the most prominent and well-characterized markers of the decidualization process (Irwin *et al.* 1994, Ramathal *et al.* 2010). By interacting mainly with the IGF1R (Wang & Chard 1999), both IGF1 and IGF2 are mitogenic factors whose uterine expression is regulated by steroidogenic hormones (De Cock *et al.* 2002, Bhatti *et al.* 2007, Dantzer & Swanson 2012). They possess differentiative properties capable of influencing embryonic development, as shown, *e.g.*, in humans and ruminants (Wathes *et al.* 1998, Irwin *et al.* 1999, Kim *et al.* 2008). Additionally, in human decidua for example, IGF1 regulates PRL and arachidonic acid secretion (Handwerger *et al.* 1991), the latter serving as a common precursor for prostaglandin synthesis.

As for the early pregnant canine uterus investigated in the present study, only the expression of IGF2 was significantly upregulated. This was concomitant with the greater abundance of *IGF2* transcripts in hatched embryos collected at days 10-12 of canine pregnancy, suggesting the predominant role of IGF2 compared to IGF1, during this very early stage of pregnancy in the dog. In contrast to the unaffected *IGF1R*-mRNA expression levels, which varied widely among individuals, expression of the respective protein was clearly detectable and seemed to be more strongly expressed during early pregnancy, implying the involvement of posttranscriptional regulatory mechanisms in its expression.

The uterine expression of PRLr, but not of PRL, was strongly upregulated after embryo exposure pre-implantation. This finding agrees with our previous report about the uterine and placental expression of PRLr (Kowalewski *et al.* 2011b), implying the possible role of PRLr-mediated effects in endometrial glandular secretory activity during the production of uterine milk (histiotrophe), a mechanism that was also suggested for humans (Jabbour *et al.* 1998). Our previous observation that interfering with progesterone receptor function by applying an antigestagen results in a decreased utero/placental PRLr expression, suggests that this involves progesterone-mediated effects (Kowalewski *et al.* 2011b). In the present study, although uterine *PRL* expression was relatively low, and frequently even below the detection limit, possible paracrine effects of locally produced PRL cannot be ruled out. Its contribution to circulating PRL levels does not seem, however, very likely. In contrast to PRLr expression, the expression of *LHR* was downregulated in the early pregnant canine uterus. Recently, the role of LHR was

suggested as a possible important factor contributing to the implantation process in mice (Gridelet *et al.* 2013). While any final conclusion concerning LHR function during the onset of canine pregnancy would be premature, we believe that this warrants further investigations.

Besides acting as one of the most potent uterotonic hormones, oxytocin also regulates secretion of other hormones, *e.g.*, prostaglandins (Meier *et al.* 1995, Fuchs *et al.* 1999). This prompted us to investigate expression of the oxytocin receptor (*OTR*) in the early pregnant uterus and its corresponding non-pregnant counterpart. Most recently (Gram *et al.* 2013b), *OTR* was localized by our group to the uterine surface epithelium of the pre-implantation uterus, specifically in the superficial and deep glands and the vascular endothelial and stromal cells. In the present study, however, *OTR* expression varied widely among individuals and, consequently, did not differ significantly between the two groups, thereby not allowing any further conclusions to be drawn regarding its potential secretory or constrictory activity during the onset of canine pregnancy. Such activity could relate, *e.g.*, to mechanisms involved in the distribution and positioning of free-floating embryos prior to attachment, or to the role of oxytocin as a mediator of local prostaglandin effects.

The expression of COX2 (PTGS2), the rate-limiting factor in the provision of prostaglandins, and of PGFS/AKR1C3 protein, was low in the early dioestric uterus and remained unaffected by the presence of embryos. The PGFS/AKR1C3 is the only canine-specific isoform of PGFS known to date and is responsible for the direct conversion of PGH2 to PGF2 α (Gram *et al.* 2013a). Since expression of the respective mRNAs was significantly increased in early pregnancy, some local effects and possible involvement of posttranscriptional regulatory mechanisms cannot be excluded, especially in view of the concomitantly increased expression of the PGF2 α -receptor (*FP*, *PTGFR*). In contrast, the expression of PGES was significantly affected both at the mRNA and protein levels in response to early embryo exposure, which together with the upregulated expression of PGT further implies local effects of prostaglandins. This conclusion also agrees with our prior report suggesting a role of prostaglandins in canine decidualization, placentation and, later on, in trophoblast invasion (Kowalewski *et al.* 2010, Gram *et al.* 2013a). The low levels of COX2 expression, together with clearly detectable uterine HPGD expression (the enzyme responsible for conversion of PGE2 and PGF2 α to their inactive metabolites) in pregnant and non-pregnant uteri, could additionally coordinate and restrict the effects of prostaglandins as local regulatory factors.

Acting through its two G protein coupled receptors designated EP2 (PTGER2) and EP4 (PTGER4), PGE2 exerts its roles mostly through the cAMP/PKA signaling pathway (Christenson *et al.* 1994, Boiti *et al.* 2001, Harris *et al.* 2001, Arosh *et al.* 2004). Also, progesterone-dependent decidualization is cAMP-mediated and this process is accelerated by PGE2 in human endometrial stromal cells (Brar *et al.* 1997). Similar effects of PGE2 and its potential to stimulate the decidual cell reaction were observed in rats (Kennedy & Doktorcik 1988). This could also be true for the canine species, as indicated in the present study by the increased uterine synthesis of PGES, EP2, and also EP4 protein, concomitant with the higher *PGES* expression in hatched embryos, possibly actively contributing to the decidualization process.

It is noteworthy that in the uterine samples investigated in the present study, derived from both early pregnant and non-pregnant bitches, stronger immunohistochemical signals were localized in the endometrial epithelial compartments. On the other hand, weaker signals were observed in the uterine stromal cells that undergo a strong, species-specific decidualization process later on in canine gestation. This, together with the unaffected *IGF1* and strongly varying *IGF1R* mRNA expression in the pre-implantation uterus, seems to be an indicator of the early stage of uterine differentiation at the beginning of pregnancy (gestational days 10-12) observed in our study. At this time, the uterine morphology is characterized by obvious embryo-induced functional changes reflected in the modified uterine milieu observed here, but does not yet exhibit the very strong structural changes related to the intense remodeling of uterine tissues that occur later in gestation, especially during decidua formation at the implantation sites. This is also indicated by the unaffected expression of *E-cadherin (CDH1)*.

Taken together with some earlier studies, our investigations describe the expression of genes that are differentially regulated in response to the presence of free-floating embryos in the uterine lumen of early pregnant dogs and provide a basis for better understanding of the uterine milieu required for proper embryo development and, thereby, for successful establishment of canine pregnancy. Elucidating possible functional interactions between these factors, e.g., their role in uterine growth and secretory activity, could be helpful in understanding some pathological conditions connected with dysregulated endocrinological responses of the uterus, that are frequently caused by impaired cross-talk between growth factors and hormones.

Declaration of interest: The authors declare that there is no conflict of interest. All authors read

and approved the final version of the manuscript.

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References:

- Amoroso EC 1952.** Placentation. In *Marshall's physiology of reproduction.*, pp. 127-316.
- Arosh JA, Banu SK, Chapdelaine P, Madore E, Sirois J & Fortier MA 2004** Prostaglandin biosynthesis, transport, and signaling in corpus luteum: a basis for autoregulation of luteal function. *Endocrinology* **145** 2551-2560.
- Baan M, Taverne MA, de Gier J, Kooistra HS, Kindahl H, Dieleman SJ & Okkens AC 2008** Hormonal changes in spontaneous and aglepristone-induced parturition in dogs. *Theriogenology* **69** 399-407.
- Beceriklisoy HB, Schafer-Somi S, Kucukaslan I, Agaoglu R, Gultiken N, Ay SS, Kaya D & Aslan S 2009** Cytokines, growth factors and prostaglandin synthesis in the uterus of pregnant and non-pregnant bitches: the features of placental sites. *Reproduction in domestic animals = Zuchthygiene* **44 Suppl 2** 115-119.
- Bhatti SF, Rao NA, Okkens AC, Mol JA, Duchateau L, Ducatelle R, van den Ingh TS, Tshamala M, Van Ham LM, Coryn M, Rijnberk A & Kooistra HS 2007** Role of progestin-induced mammary-derived growth hormone in the pathogenesis of cystic endometrial hyperplasia in the bitch. *Domestic animal endocrinology* **33** 294-312.
- Boiti C, Zampini D, Zerani M, Guelfi G & Gobbetti A 2001** Prostaglandin receptors and role of G protein-activated pathways on corpora lutea of pseudopregnant rabbit in vitro. *The Journal of endocrinology* **168** 141-151.
- Brar AK, Frank GR, Kessler CA, Cedars MI & Handwerger S 1997** Progesterone-dependent decidualization of the human endometrium is mediated by cAMP. *Endocrine* **6** 301-307.
- Buhi WC, Shille VM, Thatcher MJ, Alvarez IM & Qiu YX 1993** Identification and immunolocalization of proteins synthesized by dog endometrium and membranes. *Journal of reproduction and fertility. Supplement* **47** 141-157.
- Bukowska D, Kempisty B, Jackowska M, Wozna M, Antosik P, Piotrowska H & Jaskowski JM 2011** Analysis of integrins and vascular endothelial growth factor isoforms mRNA expression in the canine uterus during perimplantation period. *Polish journal of veterinary sciences* **14** 253-258.
- Christenson LK, Farley DB, Anderson LH & Ford SP 1994** Luteal maintenance during early pregnancy in the pig: role for prostaglandin E2. *Prostaglandins* **47** 61-75.
- Concannon P 1980** Effects of hypophysectomy and of LH administration on luteal phase plasma progesterone levels in the beagle bitch. *Journal of reproduction and fertility* **58** 407-410.
- Concannon PW, Gimpel T, Newton L & Castracane VD 1996** Postimplantation increase in plasma fibrinogen concentration with increase in relaxin concentration in pregnant dogs. *American journal of veterinary research* **57** 1382-1385.
- Concannon PW, McCann JP & Temple M 1989** Biology and endocrinology of ovulation, pregnancy and parturition in the dog. *Journal of reproduction and fertility. Supplement* **39** 3-25.
- Dantzer B & Swanson EM 2012** Mediation of vertebrate life histories via insulin-like growth factor-1. *Biological reviews of the Cambridge Philosophical Society* **87** 414-429.
- De Cock H, Ducatelle R, Tilmant K & De Schepper J 2002** Possible role for insulin-like growth factor-I in the pathogenesis of cystic endometrial hyperplasia pyometra complex in the bitch. *Theriogenology* **57** 2271-2287.

- Evans JM & Anderton DJ** 1992 Pregnancy diagnosis in the bitch: the development of a test based on the measurement of acute phase proteins in the blood. *Annls. Zootech.* **41** 397-405.
- Fuchs AR, Rust W & Fields MJ** 1999 Accumulation of cyclooxygenase-2 gene transcripts in uterine tissues of pregnant and parturient cows: stimulation by oxytocin. *Biology of reproduction* **60** 341-348.
- Gram A, Boos A & Kowalewski M.** 2013b. Uterine and placental expression of canine oxytocin receptor (OTR) during pregnancy and normal and induced parturition. In *Proceedings for 16th EVSSAR Congress*, pp. 111.
- Gram A, Buechler U, Boos A, Hoffmann B & Kowalewski MP** 2013a Biosynthesis and degradation of canine placental prostaglandins: prepartum changes in expression and function of prostaglandin F2alpha-synthase (PGFS, AKR1C3) and 15-hydroxyprostaglandin dehydrogenase (HPGD). *Biology of reproduction* **89** 2.
- Gridelet V, Tsampalas M, Berndt S, Hagelstein MT, Charlet-Renard C, Conrath V, Ectors F, Hüge F, Munaut C, Foidart JM, Geenen V & Perrier d'Hauterive S** 2013 Evidence for cross-talk between the LH receptor and LH during implantation in mice. *Reproduction, fertility, and development* **25** 511-522.
- Handwerger S, Markoff E & Richards R** 1991 Regulation of the synthesis and release of decidual prolactin by placental and autocrine/paracrine factors. *Placenta* **12** 121-130.
- Harris TE, Squires PE, Michael AE, Bernal AL & Abayasekara DR** 2001 Human granulosa-lutein cells express functional EP1 and EP2 prostaglandin receptors. *Biochemical and biophysical research communications* **285** 1089-1094.
- Hoffmann B, Hoveler R, Hasan SH & Failing K** 1992 Ovarian and pituitary function in dogs after hysterectomy. *Journal of reproduction and fertility* **96** 837-845.
- Hoffmann B, Hoveler R, Nohr B & Hasan SH** 1994 Investigations on hormonal changes around parturition in the dog and the occurrence of pregnancy-specific non conjugated oestrogens. *Experimental and clinical endocrinology* **102** 185-189.
- Irwin JC, de las Fuentes L & Giudice LC** 1994 Growth factors and decidualization in vitro. *Annals of the New York Academy of Sciences* **734** 7-18.
- Irwin JC, Suen LF, Martina NA, Mark SP & Giudice LC** 1999 Role of the IGF system in trophoblast invasion and pre-eclampsia. *Human reproduction* **14 Suppl 2** 90-96.
- Jabbour HN, Critchley HO & Boddy SC** 1998 Expression of functional prolactin receptors in nonpregnant human endometrium: janus kinase-2, signal transducer and activator of transcription-1 (STAT1), and STAT5 proteins are phosphorylated after stimulation with prolactin. *The Journal of clinical endocrinology and metabolism* **83** 2545-2553.
- Kennedy TG & Doktorcik PE** 1988 Effects of analogues of prostaglandin E2 and F2 alpha on the decidual cell reaction in the rat. *Prostaglandins* **35** 207-219.
- Kim J, Song G, Gao H, Farmer JL, Satterfield MC, Burghardt RC, Wu G, Johnson GA, Spencer TE & Bazer FW** 2008 Insulin-like growth factor II activates phosphatidylinositol 3-kinase-protooncogenic protein kinase 1 and mitogen-activated protein kinase cell Signaling pathways, and stimulates migration of ovine trophectoderm cells. *Endocrinology* **149** 3085-3094.
- Kowalewski MP, Beceriklisoy HB, Aslan S, Agaoglu AR & Hoffmann B** 2009 Time related changes in luteal prostaglandin synthesis and steroidogenic capacity during

- pregnancy, normal and antiprogesterin induced luteolysis in the bitch. *Animal reproduction science* **116** 129-138.
- Kowalewski MP, Beceriklisoy HB, Pfarrer C, Aslan S, Kindahl H, Kucukaslan I & Hoffmann B** 2010 Canine placenta: a source of prepartal prostaglandins during normal and antiprogesterin-induced parturition. *Reproduction* **139** 655-664.
- Kowalewski MP, Fox B, Gram A, Boos A & Reichler I** 2013 Prostaglandin E2 functions as a luteotrophic factor in the dog. *Reproduction* **145** 213-226.
- Kowalewski MP, Mason JI, Howie AF, Morley SD, Schuler G & Hoffmann B** 2006a Characterization of the canine 3beta-hydroxysteroid dehydrogenase and its expression in the corpus luteum during diestrus. *The Journal of steroid biochemistry and molecular biology* **101** 254-262.
- Kowalewski MP, Meyer A, Hoffmann B, Aslan S & Boos A** 2011a Expression and functional implications of peroxisome proliferator-activated receptor gamma (PPARgamma) in canine reproductive tissues during normal pregnancy and parturition and at antiprogesterin induced abortion. *Theriogenology* **75** 877-886.
- Kowalewski MP, Michel E, Gram A, Boos A, Guscetti F, Hoffmann B, Aslan S & Reichler I** 2011b Luteal and placental function in the bitch: spatio-temporal changes in prolactin receptor (PRLr) expression at dioestrus, pregnancy and normal and induced parturition. *Reproductive biology and endocrinology : RB&E* **9** 109.
- Kowalewski MP, Mutembei HM & Hoffmann B** 2008 Canine prostaglandin E2 synthase (PGES) and its receptors (EP2 and EP4): expression in the corpus luteum during dioestrus. *Animal reproduction science* **109** 319-329.
- Kowalewski MP, Schuler G, Taubert A, Engel E & Hoffmann B** 2006b Expression of cyclooxygenase 1 and 2 in the canine corpus luteum during diestrus. *Theriogenology* **66** 1423-1430.
- Luvoni GC, Chigioni S & Beccaglia M** 2006 Embryo production in dogs: from in vitro fertilization to cloning. *Reproduction in domestic animals = Zuchthygiene* **41** 286-290.
- Meier S, Lau TM, Jenkin G & Fairclough RJ** 1995 Oxytocin-induced prostaglandin F2 alpha release and endometrial oxytocin receptor concentrations throughout pregnancy in ewes. *Journal of reproduction and fertility* **103** 233-238.
- Nohr B, Hoffmann B & Steinetz BE** 1993 Investigation of the endocrine control of parturition in the dog by application of an antigestagen. *Journal of reproduction and fertility. Supplement* **47** 542-543.
- Okkens AC, Bevers MM, Dieleman SJ & Willemse AH** 1990 Evidence for prolactin as the main luteotrophic factor in the cyclic dog. *The Veterinary quarterly* **12** 193-201.
- Okkens AC, Dieleman SJ, Bevers MM, Lubberink AA & Willemse AH** 1986 Influence of hypophysectomy on the lifespan of the corpus luteum in the cyclic dog. *Journal of reproduction and fertility* **77** 187-192.
- Olson PN, Bowen RA, Behrendt MD, Olson JD & Nett TM** 1984 Concentrations of progesterone and luteinizing hormone in the serum of diestrous bitches before and after hysterectomy. *American journal of veterinary research* **45** 149-153.
- Onclin K, Silva LD, Donnay I & Verstegen JP** 1993 Luteotrophic action of prolactin in dogs and the effects of a dopamine agonist, cabergoline. *Journal of reproduction and fertility. Supplement* **47** 403-409.
- Onclin K, Verstegen JP & Concannon PW** 2000 Time-related changes in canine luteal regulation: in vivo effects of LH on progesterone and prolactin during pregnancy. *Journal of reproduction and fertility* **118** 417-424.

- Ramathal CY, Bagchi IC, Taylor RN & Bagchi MK** 2010 Endometrial decidualization: of mice and men. *Seminars in reproductive medicine* **28** 17-26.
- Schafer-Somi S, Beceriklisoy HB, Budik S, Kanca H, Aksoy OA, Polat B, Cetin Y, Ay SS & Aslan S** 2008 Expression of genes in the canine pre-implantation uterus and embryo: implications for an active role of the embryo before and during invasion. *Reproduction in domestic animals = Zuchthygiene* **43** 656-663.
- Vermeirsch H, Simoens P & Lauwers H** 2000 Immunohistochemical detection of the estrogen receptor-alpha and progesterone receptor in the canine pregnant uterus and placental labyrinth. *The Anatomical record* **260** 42-50.
- Wang HS & Chard T** 1999 IGFs and IGF-binding proteins in the regulation of human ovarian and endometrial function. *The Journal of endocrinology* **161** 1-13.
- Wathes DC, Reynolds TS, Robinson RS & Stevenson KR** 1998 Role of the insulin-like growth factor system in uterine function and placental development in ruminants. *Journal of dairy science* **81** 1778-1789.

Figure legends:

Figure 1.

Expression of insulin-like growth factor 1 (*IGF1*), *IGF2*, IGF-receptor 1 (*IGF1R*), prolactin (*PRL*) and its receptor (*PRLr*), and luteinizing hormone receptor (*LHR*) as determined by Real Time (TaqMan) PCR in uterine samples from early-pregnant uterus (pre-implantation; embryo-induced effects), and its non-pregnant counterparts. Numerical data are presented either as the mean \pm standard deviation (SD) (A,C-E), or as geometric means with deviation factor ($Xg \cdot DF^{\pm 1}$) (B, F). Bars with (*) differ at: (C) $P=0.04$, (E) $P=0.02$, (F) $P=0.01$.

Figure 2.

Expression of estrogen receptor alpha (*ER α*), *ER β* , progesterone receptor (*PGR*), oxytocin receptor (*OTR*) and *E-cadherin* (*CDH1*) as determined by Real Time (TaqMan) PCR in uterine samples from early-pregnant uterus (pre-implantation; embryo-induced effects), and its non-pregnant counterparts. Numerical data are presented either as the mean \pm standard deviation (SD) (A-C und E), or as geometric means with deviation factor ($Xg \cdot DF^{\pm 1}$) (D). Bar with (*) in (A) differ at: $P=0.03$.

Figure 3.

Expression of cyclooxygenase 2 (*COX2*, *PTGS2*), prostaglandin E2 (PGE2)-synthase (*PGES*) and of PGE2 receptors, *EP2* and *EP4* (*PTGER2* and *PTGER4*, respectively) as determined by Real Time (TaqMan) PCR in uterine samples from early-pregnant uterus (pre-implantation; embryo-induced effects), and its non-pregnant counterparts. Numerical data are presented as the mean \pm standard deviation (SD). Bars with (*) differ at: (B) $P=0.04$, (C) $P=0.02$.

Figure 4.

Expression of prostaglandin F2 α (PGF2 α)-synthase (*PGFS/AKR1C3*), PGF2 α -receptor (*FP*, *PTGFR*), prostaglandin transporter (*PGT*) and of 15-prostaglandin dehydrogenase (*HPGD*) as determined by Real Time (TaqMan) PCR in uterine samples from early-pregnant uterus (pre-implantation; embryo-induced effects), and its non-pregnant counterparts. Numerical data are presented as the mean \pm standard deviation (SD). Bars with (*) differ at: (A) $P=0.007$, (B) $P=0.02$, (C) $P=0.02$.

Figure 5.

Immunohistochemical (IHC) localization of insulin-like growth factor 2 (IGF2) (A-C) and IGF receptor 1 (IGF1R) (D,E), in early pregnant (pre-implantation) canine uterus and corresponding non-pregnant uterus. The localization of *IGF1* (F,G) and *IGF2* (H,I) mRNA expression is presented by *in situ* hybridization (ISH). Solid arrows = superficial (luminal) uterine epithelium, open arrows = superficial uterine glands, solid arrowheads = deep uterine glands, open arrowheads = myometrium. The inset to (A) shows a representative IgG isotype control for anti-rabbit immune serum.

Figure 6.

Immunohistochemical (IHC) localization of prolactin receptor (PRLr) in early pregnant (pre-implantation) canine uterus (C,D) and corresponding non-pregnant uterus (A,B). Solid arrows = superficial (luminal) uterine epithelium, open arrows = superficial uterine glands, solid arrowheads = deep uterine glands, open arrowheads = myometrium. The inset to (A) shows a representative IgG isotype control for anti-goat immune serum.

Figure 7.

Immunohistochemical (IHC) localization of progesterone receptor (PGR) (A,B), estrogen receptor alpha ($ER\alpha$) (C,D) and $ER\beta$ (E,F) in early pregnant (pre-implantation) canine uterus and corresponding non-pregnant uterus. Solid arrows = superficial (luminal) uterine epithelium, open arrows = superficial uterine glands, solid arrowheads = deep uterine glands. Insets to D and F show the myometrial expression of $ER\alpha$ and $ER\beta$, respectively. The inset to (A) shows a representative IgG isotype control for anti-mouse immune serum.

Figure 8.

Immunohistochemical (IHC) localization of cyclooxygenase 2 (COX2, PTGS2) (A), prostaglandin F2 α -synthase (PGFS/AKR1C3) (B) in early pregnant (pre-implantation) canine and of prostaglandin transporter (PGT) (C,D) and 15-prostaglandin dehydrogenase (HPGD) (E-G) in early pre-implantation uterus and corresponding non-pregnant uterus. Solid arrows =

superficial (luminal) uterine epithelium, open arrows = superficial uterine glands, solid arrowheads = deep uterine glands. Insets in B, D and G show the myometrial expression of PGFS/AKR1C3, PGT and HPGD, respectively.

Figure 9.

Immunohistochemical (IHC) localization of prostaglandin E2 - synthase (PGE2-synthase, PGES) (A,B), PGE2-receptors EP2 (PTGER2) and EP4 (PTGER4) (C,D) and (E,F), respectively, in the early pregnant (pre-implantation) canine uterus and corresponding non-pregnant uterus. Solid arrows = superficial (luminal) uterine epithelium, open arrows = uterine glands. Insets to B, D and F show the myometrial expression of PGES, EP2 and EP4, respectively. The inset to (A) shows a representative IgG isotype control for anti-guinea pig immune serum.

Figure 10.

Embryonal expression of prostaglandin E2-synthase (*PGES*), cyclooxygenase 2 (*COX2*, *PTGS2*), insulin-like growth factor 1 (*IGF1*) and *IGF2* as determined by Real Time (TaqMan) PCR.

Tables:

Table 1.

List of primers and TaqMan Probes used for the semi-quantitative RT-PCR

Table 2.

List of antibodies used for the immunohistochemistry

Real Time PCR

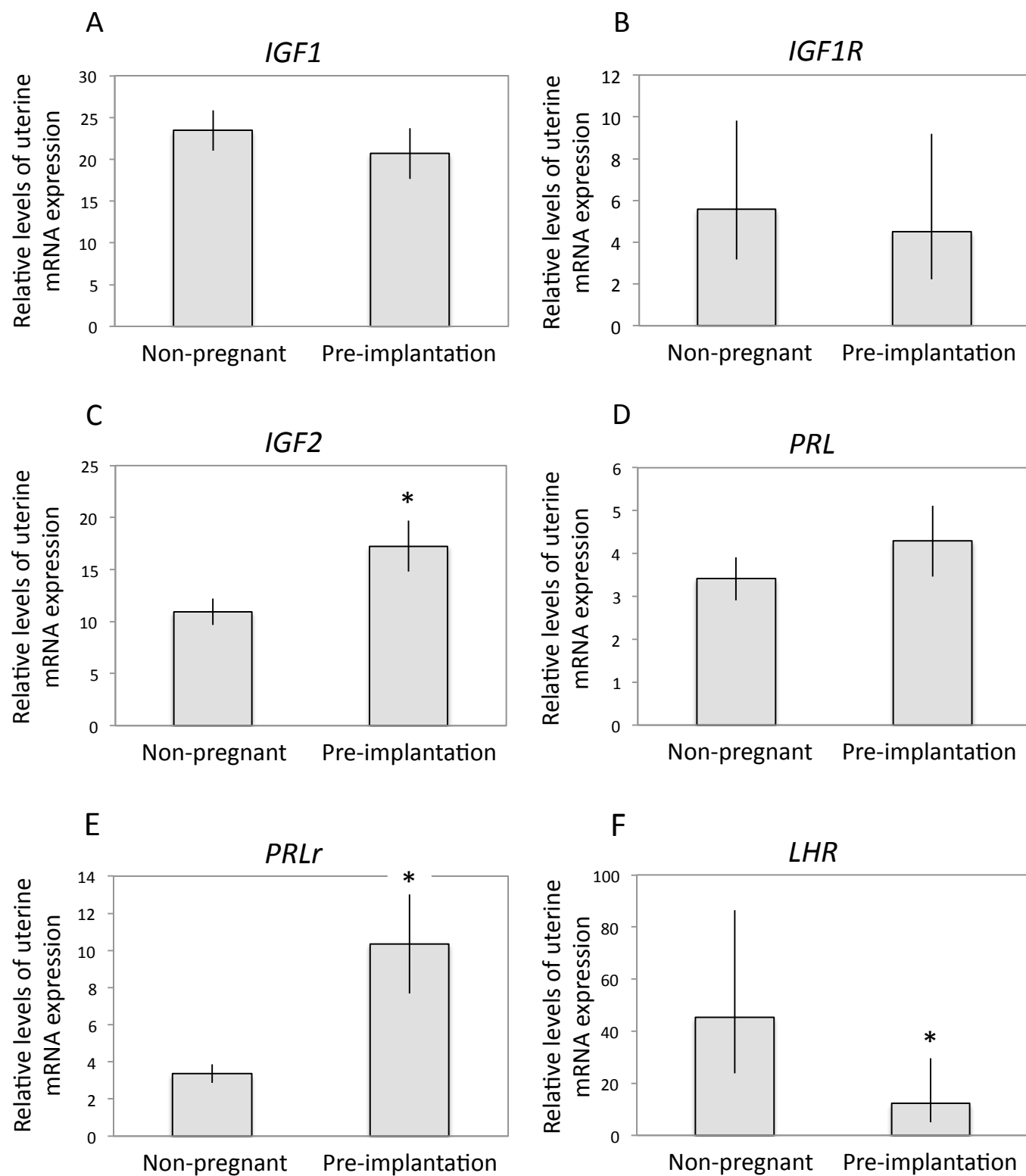


Figure 1

Real Time PCR

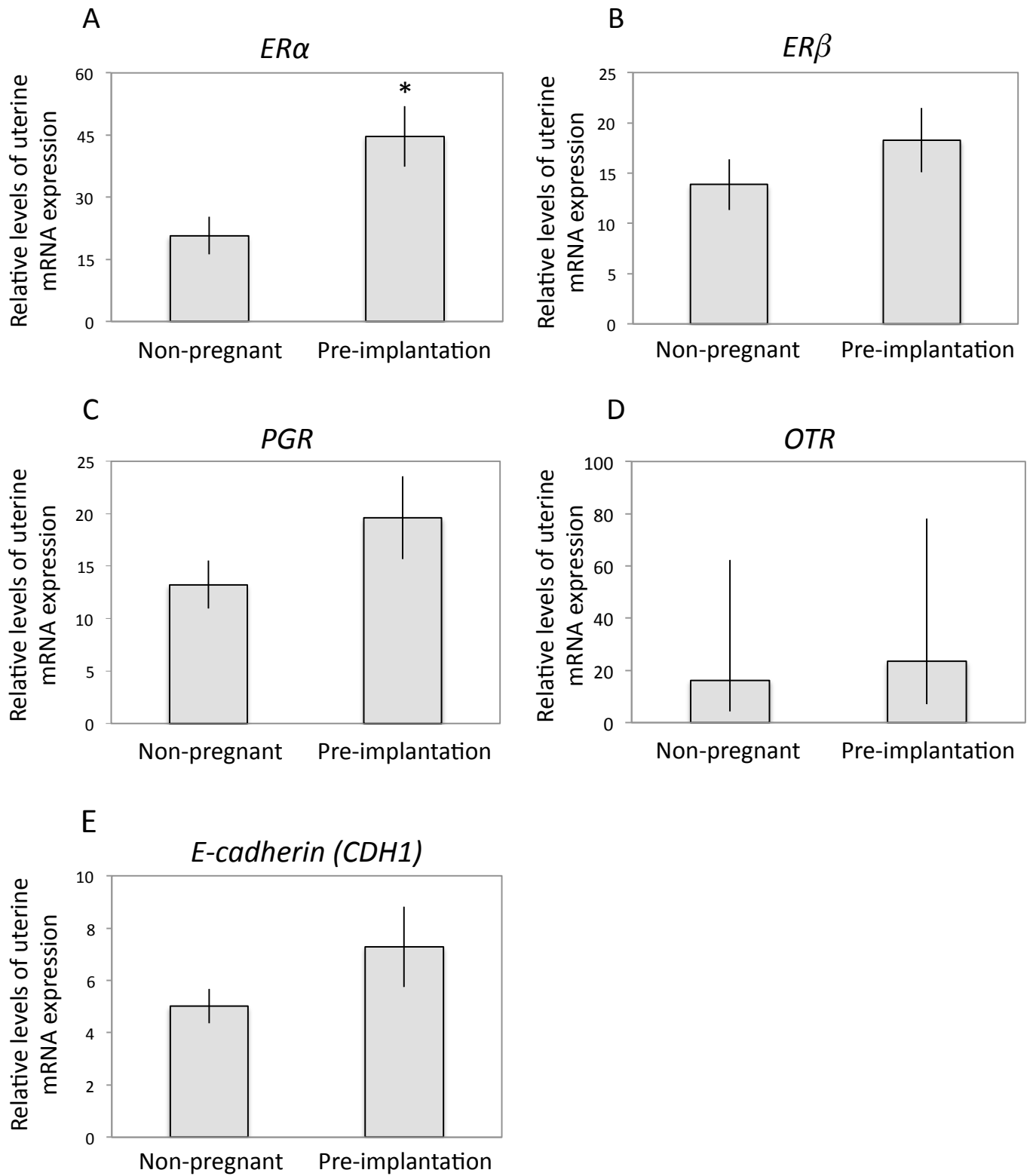


Figure 2

Real Time PCR

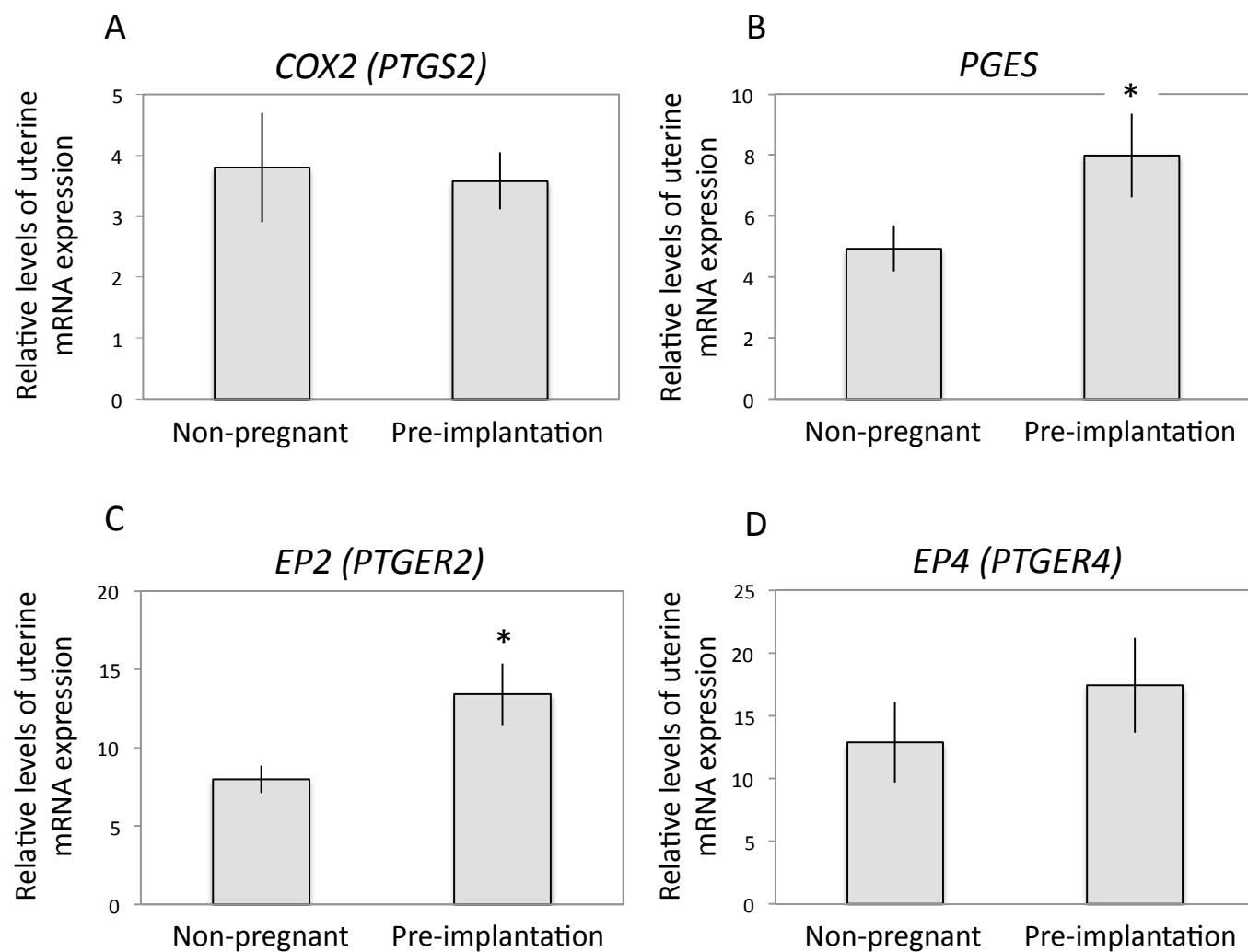


Figure 3

Real Time PCR

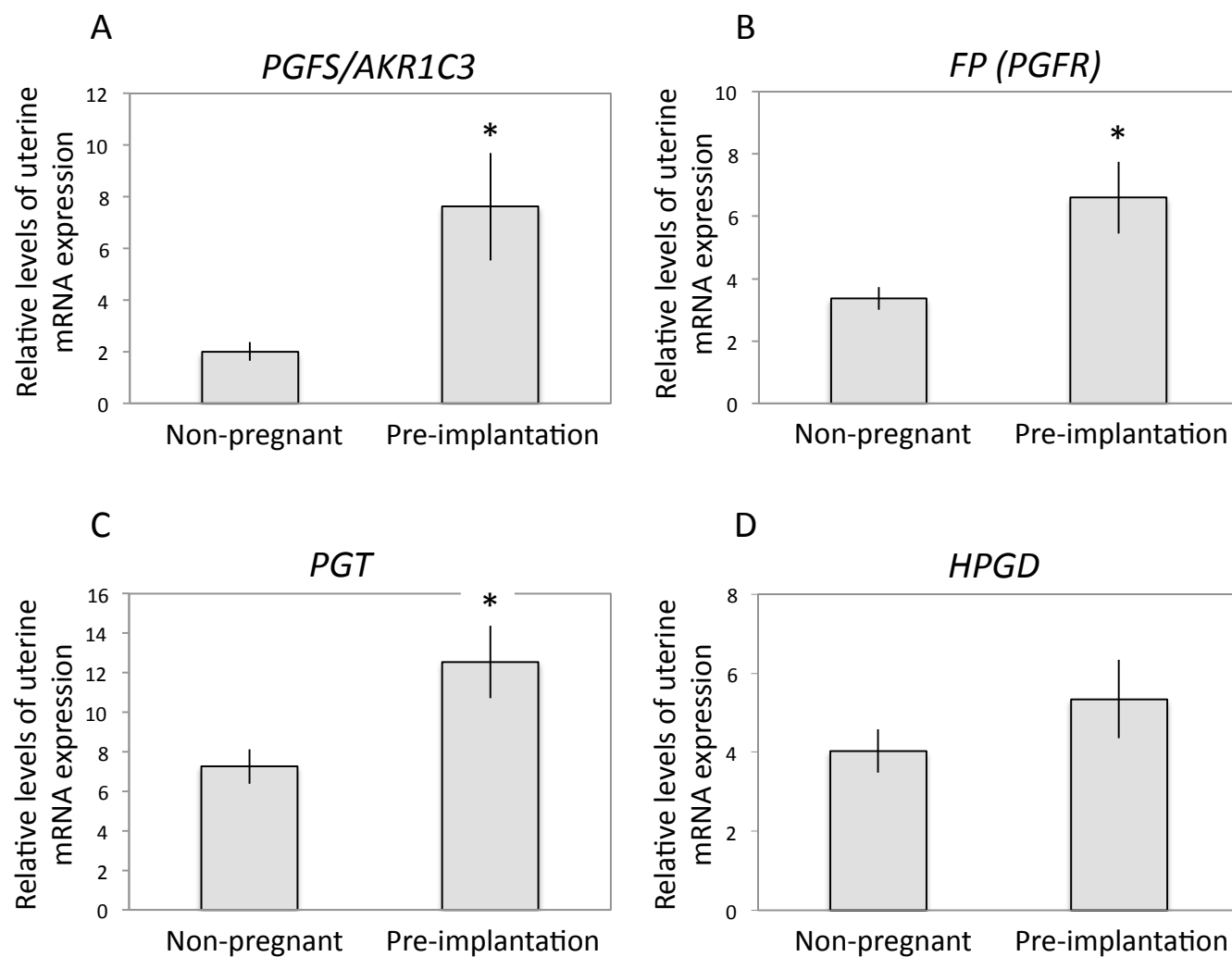


Figure 4

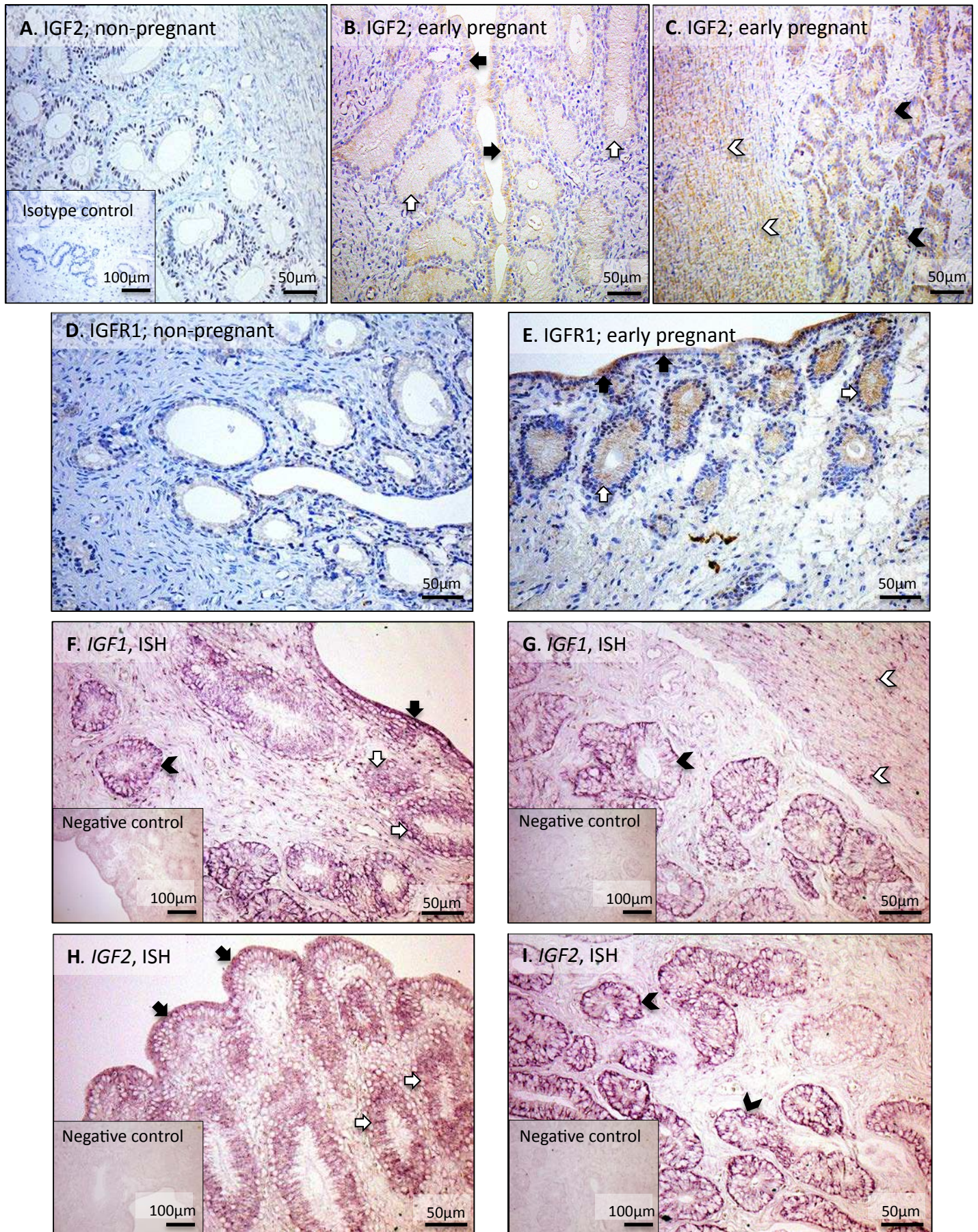


Figure 5

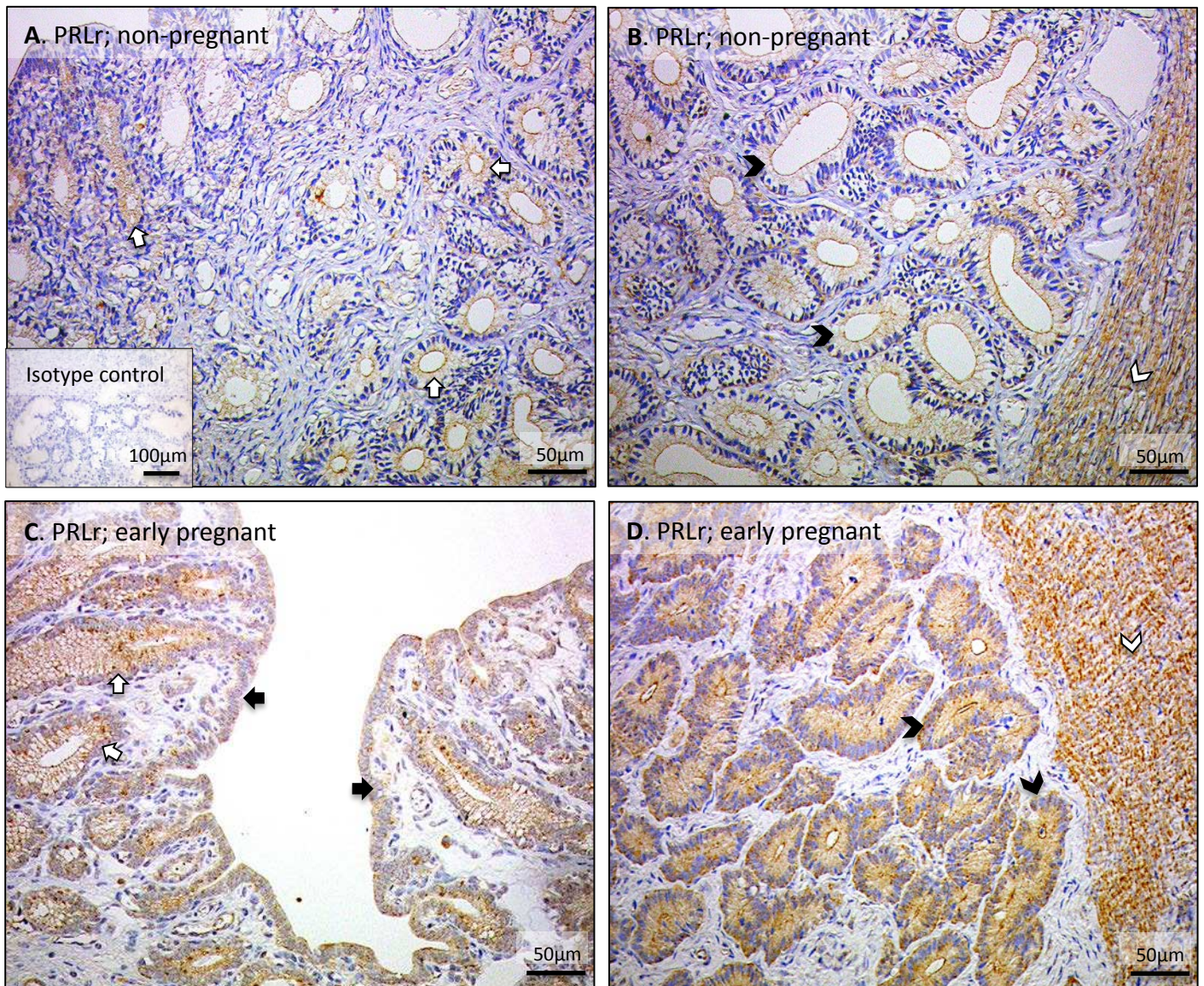


Figure 6

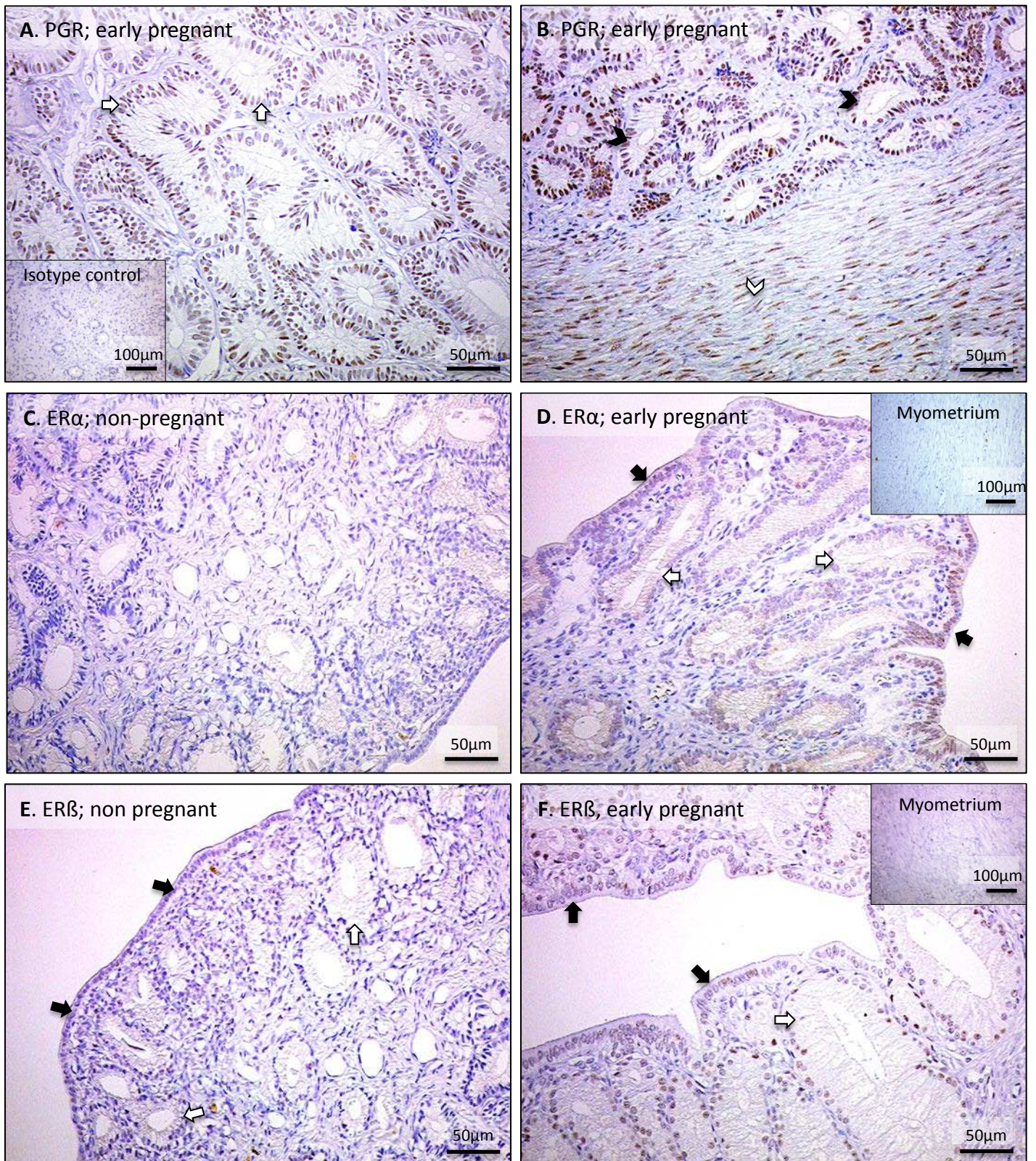


Figure 7

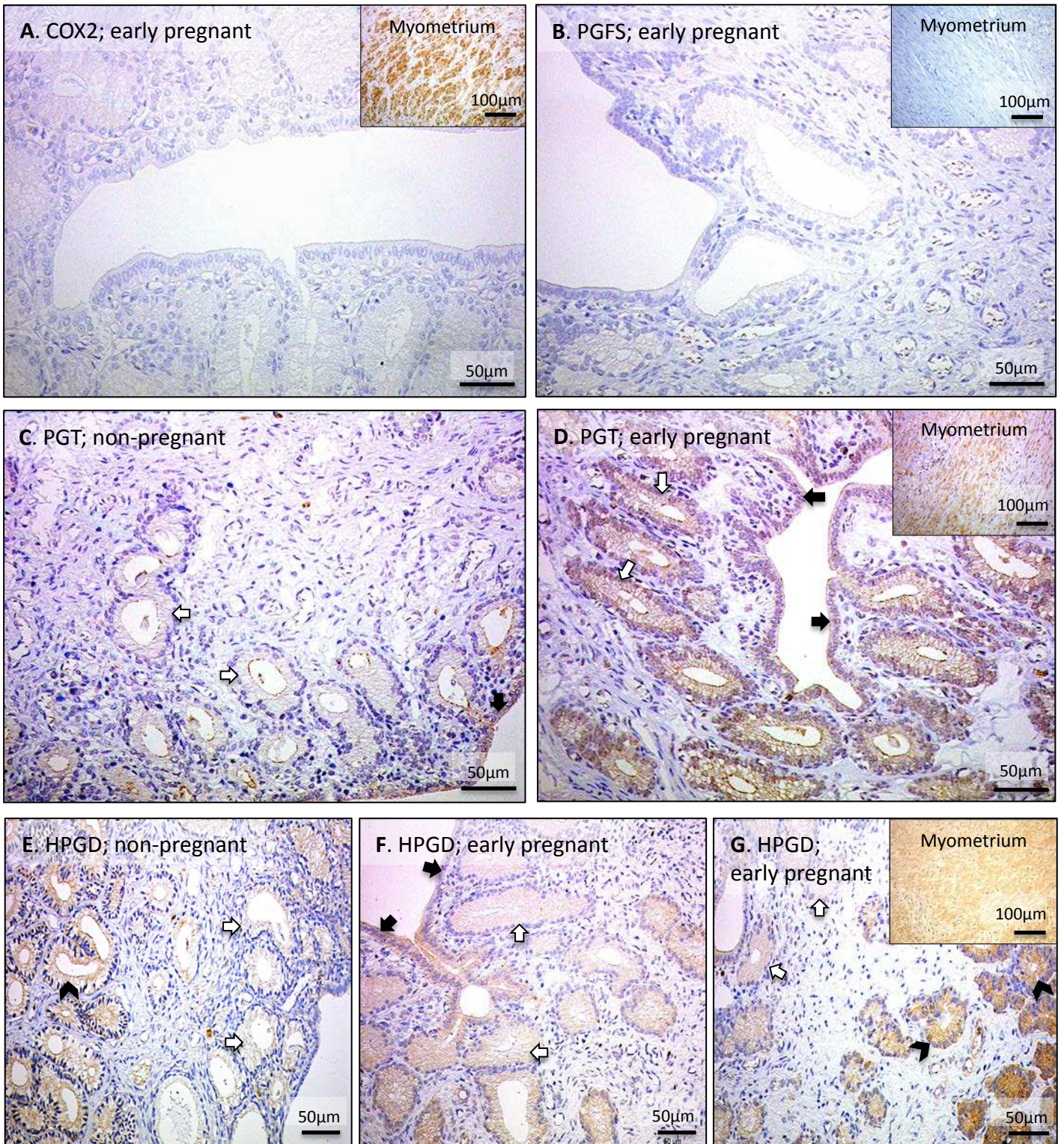


Figure 8

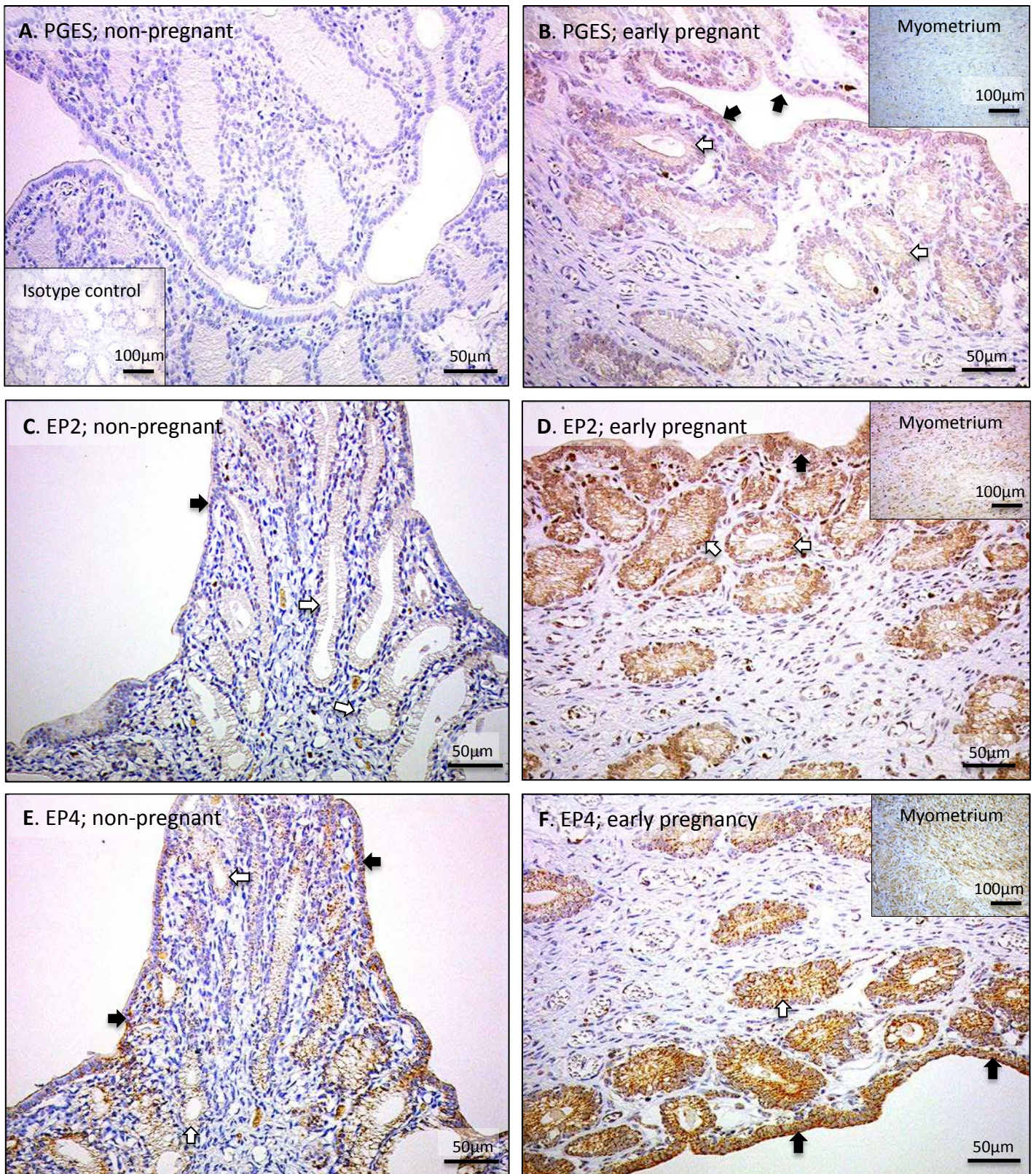
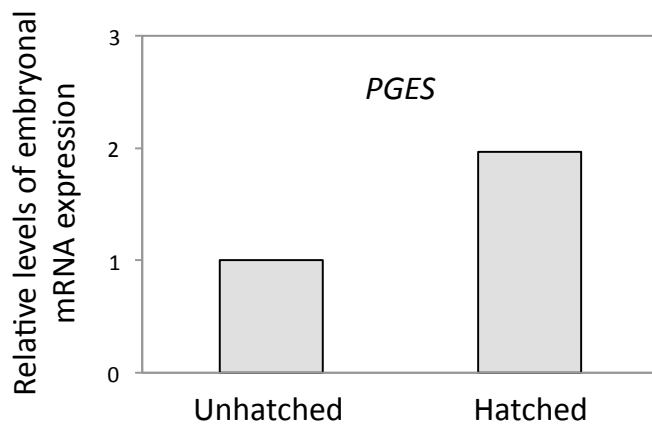


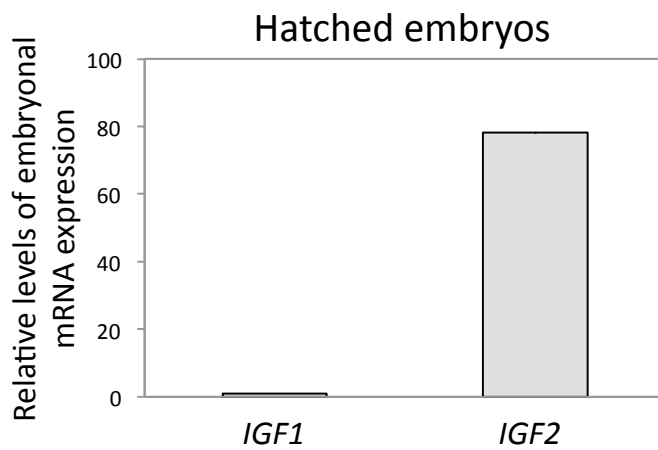
Figure 9

Real Time PCR

A



B



C

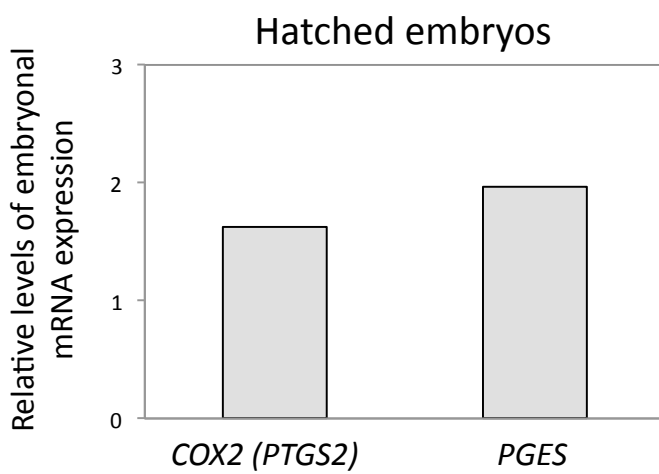


Figure 10

Primer	Accession numbers	Primer Sequence	Product lenght (bp)
GAPDH (forward) GAPDH (reverse) GAPDH (TaqMan Probe)	AB028142	5'-GCT GCCAAATAT GACGACATC A-3' 5'-GTA GCC CAG GAT GCC TTT GAG-3' 5'-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3'	75bp
18SrRNA (forward) 18SrRNA (reverse) 18SrRNA (TaqMan Probe)	FJ797658	5'-GTC GCT CGC TCC TCT CCT ACT-3' 5'-GGC TGA CCG GGT TGG TTT-3' 5'-ACA TGC CGA CGG GCG CTG AC-3'	125bp
IGF1R (forward) IGF1R (reverse) IGF1R (TaqMan Probe)	XM545828	5'-GGA CGT TGA GCC TGG CAT T-3' 5'- CAC TCT TAG CCC CAC GGA TGT-3', 5'- AGC CCT GGA CGC AGT ATG CGG -3	119bp
PRLr (forward) PRLr (reverse) PRLr (TaqMan Probe)	HQ267784	5'-GGA TCT TTG TGG CCG TTC TTT-3' 5'-AAG GAT GCA GGT CAC CAT GCT AT-3' 5'-ATT ATG GTC GTA GCA GTG GCT TTG AAA GGC-3'	92bp
PRL (forward) PRL (reverse) PRL (TaqMan Probe)	NM_00108275	5'-CAA GCC CAA CAG ATC CAC CAT-3' 5'-ATC CCC CGC ACT TCT GTG A-3' 5'- CTG AGG GTG CTG CGC TCC TGG -3'	104bp
PGR (forward) PGR (reverse) PGR (TaqMan Probe)	NM_001003074	5'-CGA GTC ATT ACC TCA GAA GAT TTG TTT -3' 5'- CTT CCA TTG CCC TTT TAA AGA AGA-3' 5'- AAG CAT CAG GCT GTC ATT ATG GTG TCC TAA CTT-3'	113bp
ERα (forward) ERα (reverse) ERα (TaqMan Probe)	XM533454	5'-CCC ATG GAG GAG ACA AAC CA -3' 5'-CCC TGC CTC CTC GGT GAT ATA -3' 5'-CAC GGG CCC AAC TTC ATC ACA TTC C -3'	93bp
ERβ (forward) ERβ (reverse) ERβ (TaqMan Probe)	XM861041	5'-CCC AGC ACG CCC TTC A -3' 5'-AAT CAT ATG CAC GAG TTC CTT GTC -3' 5'-CCT CCA TGA TGA TGT CCC TGA CC-3'	78bp
COX2 (forward) COX2 (reverse) COX2 (TaqMan Probe)	HQ110882	5'- GGA GCA TAA CAG AGT GTG TGA TGT G-3' 5'- AAG TAT TAG CCT GCT CGT CTG GAA T-3' 5'- CGC TCA TCA TCC CAT TCT GGG TGC -3'	87bp
PGFS (forward) PGFS (reverse) PGFS (TaqMan Probe)	NM_001012344	5'-AGG GCT TGC CAA GTC TAT TGG -3' 5'-GCC TTG GCT TGC TCA GGA T -3' 5'- TCC AAC TTT AAC CGC AGG CAG CTG G-3'	74bp
PGES (forward) PGES (reverse) PGES (TaqMan Probe)	NM_001122854	5'-GTC CTG GCG CTG GTG AGT -3' 5'- ATG ACA GCC ACC ACG TAC ATC T-3' 5'-TCC CAG CCT TCC TGC TCT GCA GC -3'	89bp
PTGFR (FP) (forward) PTGFR (FP) (reverse) PTGFR (FP) (TaqMan Probe)	NM_001048097	5'- ACC AGT CGA ACA TCC TTT GCA-3' 5'- GGC CAT CAC ACT GCC TAG AAA-3' 5'-CAT GGT GTT CTC CGG TCT GTG CCC -3'	86bp
PTGER2 (forward) PTGER2 (reverse) PTGER2 (TaqMan Probe)	AF075602	5'- CAC CCT GCT GCT GCT TCT C-3' 5'- CGG TGC ATG CGG ATG AG-3' 5'-TGC TCG CCT GCA ACT TTC AGC GTC -3'	78bp
PTGER4 (forward) PTGER4 (reverse) PTGER4 (TaqMan Probe)	NM_001003054	5'- AAA TCA GCA AAA ACC CAG ACT TG-3' 5'-GCA CGG TCT TCC GCA GAA -3' 5'-ATCCGA ATT GCT GCT GTG AAC CCT ATC C -3'	96bp
PGT (forward) PGT (reverse) PGT (TaqMan probe)	NM_001011558	5'-TGC AGC ACT AGG AAT GCT GTT C-3' 5'-GGG CGC AGA GAA TCA TGG A-3' 5'-TCT GCA AAC CAT TCC CCG CGT G-3'	116bp
HPGD (forward) HPGD (reverse) HPGD (TaqMan Probe)	NM_001284477	5'-GGC AGC GAA TCT CAT GAA CAG-3' 5'- TCT TCT TTC TCA ATG GAT TCA AGGA-3' 5'- TGA ATG CCA TTT GCC CAG GCT TTG-3'	93bp
OXTR (forward) OXTR (reverse) OXTR (TaqMan Probe)	NM_001198659	5'- GGA TCA CGC TCT CCG TCT ACA-3' 5'-CGT CTT GAG TCG CAG GTT CTG-3' 5'- CCT GCT ACG GCC TCA TCA GCT TCA A-3'	98bp
LHR (forward) LHR (reverse) LHR (TaqMan Probe)	XM538486	5'- TCA TCA TTT GTG CTT GCT ACA TTA AA-3' 5'- CGC CAT TTT CTT AGC AAT CTT TG-3' 5'- TGC AGT TCA AAA TCC AGA GCT GAT GGC-3'	98bp

Table 1

Name/Antigen	Clone	Company	Immunogen	Concentration	Species / Type
IGFR1	Ibs-0227R	Bioss Inc. MA, USA	Ab against human IGFR1	1:800	rabbit polyclonal
IGF2	IGF-II (H103) Sc-5622	Santa Cruz Biotechnology CA, USA	Ab against human IGF-2	1:200	rabbit polyclonal
PGR	clone 10A9	Immunotech Hamburg Germany	Ab against human C-terminal PGR	1:100	mouse monoclonal
ERα	NCL-ER-6F11	Novo Castra New Castle UK	Ab against human ER α	1:10	mouse monoclonal
ERβ	MCA 1974	Serotec Germany	Ab against human ER β	1:20	mouse monoclonal
PRLr	catalog no. AF 1167	R&D Systems Europe Ltd.	NSO-derived rhProlactin R extracellular domain	1:50	goat polyclonal
COX2	clone 33	BD Pharmingen Heidelberg Germany	anti-rat COX-2 IgG	1:100	mouse monoclonal
PGT	(G-17) Sc-103085	Santa Cruz Biotechnology CA, USA	IgG against human PGT	1:100	goat polyclonal
PGFS/AKR1C3	custom made canine-specific antibody (Gram et al., 2013)	Eurogentec Seraing Belgium	IgG against canine-specific peptide sequence DTLFATHPDYPFNDED, C-terminal amino acids 309-324	1:750	guinea pig polyclonal
PGES	custom made canine-specific antibody	Eurogentec Seraing Belgium	IgG against canine-specific peptide sequence RSDQDVDRCLRAHRND, C-terminal amino acids 61-76	1:300	guinea pig polyclonal
HPGD	custom made canine-specific antibody (Gram et al., 2013)	Eurogentec Seraing Belgium	IgG against canine-specific peptide sequence HFQDYETTPFHAKTQ, C-terminal amino acids 252-266	1:750	guinea pig polyclonal
EP2	catalog no. 101770	Cayman Chemicals MI, USA	IgG against human EP2, C-terminal amino acids 335-358	1:200	rabbit polyclonal
EP4	catalog no. 101775	Cayman Chemicals MI, USA	IgG against human EP4, C-terminal amino acids 459-488	1:100	rabbit polyclonal
Isotype control	IgG	Vector Laboratories Inc., Burlingame, CA, USA	-	Same protein concentration as primary antibody	mouse
Isotype control	IgG	Vector Laboratories Inc., Burlingame, CA, USA	-	Same protein concentration as primary antibody	rabbit
Isotype control	IgG	Vector Laboratories Inc., Burlingame, CA, USA	-	Same protein concentration as primary antibody	goat
Isotype control	IgG	Vector Laboratories Inc., Burlingame, CA, USA	-	Same protein concentration as primary antibody	guinea pig

Table 2